Disjunct populations of *Monocarpus sphaerocarpus* (Monocarpaceae, Marchantiopsida) within Australia show no sequence variation

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Introduction

Monocarpus sphaerocarpus D.J.Carr is a relatively rare and intriguing Southern Hemisphere liverwort. With extant plants known only from a few locations in Victoria, South Australia and Western Australia (Fig. 1), it is typically found growing on subsaline soils around saline lakes or near coastlines (Figs 2a, b). When initially described by Carr in 1956, its placement within the liverworts was not entirely clear (Carr 1956). Carr considered it closely related to *Sphaerocarpos* Boehm. and raised a new suborder Monocarpineae to accommodate the new species but argued that *Monocarpus* D.J.Carr, *Sphaerocarpos* and allied genera should be placed within the order Marchantiales. Subsequent authors have debated its position (see summary in Forrest et al. 2015). It was not until molecular data became available that this species was finally confirmed to be sister to members of the Sphaerocarpaceae, supporting its place within the Order Sphaerocarpales (Forrest et al. 2015).

Abstract

Populations of the complex thalloid liverwort *Monocarpus sphaerocarpus* D.J.Carr (Monocarpaceae) found in a disjunct distribution across southern Australia were sequenced to investigate whether plants found in eastern Australia were a different species from those found on the west coast of Australia, in line with potential differences in spore morphology. Sequences from five plastid gene regions were generated from specimens collected from several populations found in the states of Victoria, South Australia and Western Australia and spore morphology was reviewed using light and scanning electron microscopy. All individuals belonged to a single haplotype on the basis of the five plastid markers *psb*A-trnH, *trn*L-F, *rbc*L, *rpo*C1, and *rps*4-trnAS. Moreover, observed morphological differences were not consistent with geographical provenance and more likely indicative of infraspecific variation or difference in spore maturity when collected. These results support the recognition of only a single species in Australia.

Key words: liverworts, plastid markers, invariant loci, spore

In this same study, Forrest et al. (2015) compared spores from Australian populations with spores from the single known South African collection of the species (Perold 1999), finding distinct differences between the South African and Australian plants. Forrest et al. (2015) also suggested that plants from populations in Western Australia may not be conspecific with those in Victoria, based on spore differences: the cingulum is more pronounced in the Victorian populations than that of the Western Australian population (Figs 2d, e). Moreover, the disjunct distribution was cited as possible evidence for the recognition of two different species (Forrest et al. 2015). In our study, several Australian populations were sampled in the eastern, southern and western parts of the country, and five molecular markers were sequenced from these samples in order to investigate the delimitation of the taxa.

Materials and methods

Molecular data: DNA extraction, amplification and sequencing

Samples of *Monocarpus sphaerocarpus* were obtained from a number of recent collections from previously known localities in Western Australia and Victoria, as well as from two new localities in South Australia (see Table 1). In total, 12 new samples were collected (five from Western Australia, five from Victoria and two from South Australia) and compared to a previously studied specimen from Western Australia (Forrest et al. 2015). DNA extractions were performed using a Qiagen DNeasy Plant Mini kit following the manufacturer's instructions (Qiagen, Melbourne, Victoria, Australia). Five plastid gene regions were amplified: *rbc*L, *rpo*C1,

Figure 1. Map of known locations of *Monocarpus sphaerocarpus* in Australia, indicated by red filled circles, including locations of populations sampled for this study as blue filled circles

Table 1. Taxon and gene sampling for this study. Gen8ank numbers are listed for each marker and missing data are represented by a dash. GenBank numbers shown in bald were newly produced as part of this study.

Species	Coll. number (herbarium)	DNA voucher	Locality details	rbcL	rpoC1	trnL-F	psbA-trnH	rps4-trnAS
Manacarpus sphaeracarpus	HMJ171-1 (CAN8)	HMJ171-1	Australia, WA, at the edge af Yarra Yarra Lakes	KY707128	KY707149	KY707155	KY707164	KY707139
Manacarpus sphaerocarpus	HMJ171-2 (CAN8)	HMJ171-2	Australia, WA, at the edge of Yarra Yarra Lakes	KY707129	KY707150	KY707156	KY707165	KY707140
Manacarpus sphaeracarpus	HMJ172 (MEL)	HMJ172	Australia, WA, at the edge af Yarra Yarra Lakes	KY707130	–	KY707157	KY707166	KY707141
Manacarpus sphaeracarpus	HMJ173 (MEL)	HMJ173	Australia, WA, at the edge of Yarra Yarra Lakes	KY707131	–	–	KY707167	–
Manacarpus sphaerocarpus	HMJ174 (MEL)	HMJ174	Australia, WA, at the edge of Yarra Yarra Lakes	KY707132	–	KY707160	–	KY707142
Manacarpus sphaerocarpus	Jalley & Milne 171 (E)	–	Australia, WA, at the edge of Yarra Yarra Lakes	KT356975	KT357003	–	KT356965	KT356985
Manacarpus sphaerocarpus	JM606 (MEL)	JM606	Australia, VIC, red ochre pit, Raak Plain	KY707133	–	–	KY707168	KY707145
Manacarpus sphaeracorpus	JM607 (MEL)	JM607	Australia, VIC, old gypsum mine, appasite red ochre pit, Raak Plain	KY707134	KY707151	KY707161	KY707169	–
Manacarpus sphaeracarpus	JM611 (MEL)	JM611	Australia, VIC, Lake Duchembegarra Flara and Fauna Reserve	KY707135	KY707152	–	KY707170	KY707146
Manacarpus sphoeracarpus	JM605 (CAN8)	JM605	Australia, VIC, Nawingi Line Track, near Raak Plain	–	–	KY707159	KY707171	KY707144
Manacarpus sphaerocarpus	JM605-2 (CAN8)	JM605-2	Australia, VIC, Nowingi Line Track, near Raak Plain	KY707136	–	KY707158	KY707172	KY707143
Manacarpus sphaeracarpus	DCC13535A (CAN8)	DCC1353SA	Australia, 5A, Winninawie Canservation Park	KY707137	KY707153	KY707162	–	KY707147
Manacorpus sphoeracarpus	DCC13555A (CANB)	DCC1355SA	Australia, 5A, Part Augusta, on mud flats	KY707138	KY707154	KY707163	KY707173	KY707148
Sphaeracarpas drewei	Dayle 7193 (E)	07-02394	U5A, Califarnia	KY823573	KY823561	–	KY823485	–
Sphaeracarpas drewei	5chill 142 (no. 233) (E)	08-01271	U5A, Califarnia	KY823573	KY823561	–	KY823486	–
Sphaeracarpas michelii	Thauvenat s.n. (H918)	06-06279	France	KY823573	KY823561	–	KY823487	–
Sphaerocarpas michelii	Long 36568 (E)	08-00257	Partugal	KY823573	KY823561	–	KY823488	–
Sphaerocarpas michelii	Halyaak 03-417 (E)	08-00485	Great-Britain, Carnwall	KY823573	KY823561	–	KY823489	–
Sphaerocarpas michelii	Lang 36581-a (E)	08-00495	Partugal	KY823573	KY823561	–	KY823490	–
Sphaeracarpas stipitatus	Heddersan 16432 (E)	07-01921	5auth-Africa, Cape	KY823573	KY823561	–	KY823491	–
Sphaerocarpas stipitatus	Long 20843 (E)	07-02112	Nepal	KY823573	KY823561	–	KY823492	–
Sphaeracarpas texanus	Thauvenat s.n. (H917) (E)	06-06278	France	KY823573	KY823561	–	KY823493	–
Sphaeracarpos texanus	Lang 36573 (E)	07-01671	Partugal, Beira Litoral	KY823573	KY823561	–	KY823494	–
Sphaeracorpas texanus	Ghullam TG1868439155 (E)	08-01275	Great-8ritain, Narfalk	KY823573	KY823561	–	KY823495	–
Sphaeracarpas texanus	Etdaej s.n. (E)	08-01287	Turkey	KY823573	KY823561	–	KY823496	–

trnL-F, psbA-trnH and rps4-trnAS. Some DNA sequence data was also generated for accessions from four species of *Sphaerocarpos*, for the restricted set of plastid regions, rpoC1, rbcL and psbA-trnH (Table 1). Primer sequences and amplification parameters are shown in Table 2. For the amplification reactions, 1 µl of genomic DNA was added to the following PCR mix: 2.5 µl 10 × NH$_4$ PCR buffer, (Bioline, London, UK); 1.25 µl of MgCl$_2$ (50 mM); 2.5 µl dNTP (2 mM); 1 µl of each primer (10 µM); 0.3 µl DNA polymerase Bioline BioTaq (5 U/µl^{-1}), and water to a total volume of 25 µl. The PCRs were performed using a Mastercycler pro S (Eppendorf, Hamburg, Germany). The PCR products were sent to Macrogene (Seoul, South Korea) for cleaning and sequencing. Sequences were assembled and edited using Sequencher v. 5.4.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA) and aligned manually with Mesquite v. 3.04 (Maddison & Maddison 2016) and inspected visually. GenBank numbers are available in Table 1.

Morphology: scanning electron and light microscope images of spores

Mature or near mature spores were taken from fertile plants and placed in water on glass slides for light microscopy. Photos of the habitat and plants *in situ* were taken with a Canon Powershot G12 digital camera and light micrographs were taken with a Nikon Coolpix 500 digital camera. For scanning electron microscopy (SEM), spores were placed on double-sided sticky tape on aluminium stubs, coated with gold and viewed using a Zeiss EVO LS 15 Environmental SEM.

Results

Molecular data

Newly obtained sequences and sequences already available in GenBank were aligned manually and compared visually. Eleven new sequences were obtained for rbcL (Table 1). They were aligned together with the rbcL sequence of *Monocarpus sphaerocarpus* available from GenBank (KT356975), but show no variation among the 593 overlapping nucleotide sites. Similarly, the six new *Monocarpus* sequences of rpoC1 (797 nucleotide sites) were identical, and identical to the rpoC1 sequence available in GenBank for *M. sphaerocarpus* (KT357003). All nine new *Monocarpus* sequences of trnL-F (456 nucleotide sites) were the same. No variation was seen between ten new sequences of

Table 2. List of PCR primers used to amplify the five markers psbA-trnH, rbcL, rpoC1, rps4-trnAS and trnL-F. PCR conditions are described as follows: initial denaturation, number of cycles × (denaturation, annealing, extension), final extension.

Gene region	Direction	Primer name	Primer sequence	Authors	PCR conditions
psbA-trnH	forward	psbA-501F	TTTCTCAGACGGTATGCC	C. Cox, Duke, NC, pers. comm.	94°C (1 min), 35x [93°C (1 min), 50°C (1 min), 72°C (3 min)], 72°C (7 min)
	reverse	trnHR	GAACGACGGGAATTGAAC	C. Cox, Duke, NC, pers. comm.	
rbcL	forward	rbcL-aaf	ATGTCACCACAAACAGAGACTAAAGC	Kress & Erickson 2007	94°C (1 min), 35x [93°C (1 min), 50°C (1 min), 72°C (3 min)], 72°C (7 min)
	reverse	rbcL-aar	CTTCTGCTACAAATAAGAATCGATCTC	Kress & Erickson 2007	
	reverse	rbcL-634R	GAAACGGTCTCTCCAACGCAT	Fazekas et al. 200B	
rpoC1	forward	rpoC1-LP1	TATGAAACCAGAATGGATGG	Kew website as cited by Wynns & Asmussen-Lange 2014	94°C (1 min), 40x [94°C (30 sec), 48°C (40 sec), 72°C (40 sec)], 72°C (5 min)
	reverse	rpoC1-LP5	CAAGAAGCATATCTTGASTYGG	Kew website as cited by Wynns & Asmussen-Lange 2014	
rps4-trnAS	forward	rps5	ATGTCCCGTTATCGAGGACCT	Nadot et al. 1994	94°C (1 min), 35x [93°C (1 min), 50°C (1 min), 72°C (3 min)], 72°C (7 min)
	reverse	trnAS	TACCGAGGGTTCGAATC	Baker pers. comm. in Cox et al. 2000	
trnL-F	forward	trnLF-c	CGAAATCGGTAGACGCTACG	Taberlet et al. 1991	94°C (1 min), 35x [93°C (1 min), 50°C (1 min), 72°C (3 min)], 72°C (7 min)
	reverse	trnLF-f	ATTTGAACTGGTGACACGAG	Taberlet et al. 1991	

*rps*4-trnAS (1034 nucleotide sites), or between those and the *rps*4-trnAS sequence available in GenBank for *M. sphaerocarpus* (KT356985). Finally, no variation was found between ten new *Monocarpus* sequences of *psb*A-trnH (645 nucleotide sites), or between those and the *psb*A-trnH sequence available in GenBank for *M. sphaerocarpus* (KT356965). Obtained *psb*A-trnH sequences were short as the reaction for the reverse primer failed to sequence for nine out of ten samples. Our *psb*A-trnH sequences are, therefore, based on the single strand of the forward primer. A repeat at the 3′ end of *psb*A-trnH might have cause the sequencing problem. Although data were missing for all five markers (one sequence for *rbcL*, six for *rpoC1*, four for *trnL*-F, two for *psb*A-trnH and two for *rps*4-trnAS), sequences obtained came from specimens collected in all three sites: Western Australia, Victoria and South Australia. Based on our taxon sampling, none of these markers shows any variation across the Australian distribution range of the species (from Western Australia to Victoria).

Spore morphology

The spores of *Monocarpus sphaerocarpus* are more or less hemispherical in equatorial view, between 42–60 μm in diameter and 40–50 μm in height. The distal face is distinctly spinose to tuberculate (Figs 2c, e, g). The proximal face is without a triradiate mark, may be flat or concave in equatorial view, and with a structure not unlike a short cylinder projecting from the proximal face (Fig. 2f). On the proximal surface runs a cingulum which is almost doughnut-shaped; within the 'hole', or central area of the cingulum, are a series of hole-like depressions (Figs 2d, e). The spores from all populations were re-examined and ornamentation patterns found to be more or less the same for the convex distal face, which in all cases consists of numerous spine-like or peg-like protuberances (Figs 2c, e). The variation between Victorian and Western Australian populations, as also noted by Forrest et al. (2015), related to the prominence of the proximal cingulum which surrounds the central portion of the proximal face. In equatorial view, this part of the proximal face projects as a short solid cylinder surrounding the flattened to slightly concave central part of the face (Fig. 2f). In proximal view, this central area is ornamented with irregular vermiform ridges and tubercles that form small, irregular holes or

areolae. Spores from the Victorian populations have prominent cingula which are more or less smooth (Fig. 2d), whereas the cingula of Western Australian populations are variable with some spores in the same capsule with smooth cingula similar to those seen in the Victorian populations (Fig. 2g). In some spores, the cingulum is not as prominent or smooth but instead has raised uneven protuberances, making it more textured in appearance (Fig. 2e). However, spores have also been found within the Western Australian populations that possess the more prominent smooth cingulum, approaching those seen in the Victorian spores. The South Australian spores are more similar to the Western Australian spores, with a less prominent cingulum, but this too can vary. Measurements and observations were made on two collections from Victoria (*Carr s.n.* Nangiloc, Vic & *Carr s.n.* Salt Lake nr Nowingi, Vic, both at CANB), which had open mature capsules, and one from Western Australia (*HMJ 171* at CANB), which had capsules that are shrunken and intact and therefore immature. The diameter range of the mature Victorian spores is 52.5–65 μm while those from the Western Australian collections range from 37.5–55 um, considerably smaller. Cingula are well developed and prominent in equatorial views of the Victorian spores, while the cingula of the Western Australian spores have not developed at all. This observed variation amongst populations can be due to natural variation within a capsule, or amongst individuals within a population. Given that capsules of different ages were compared, the observed differences are explicable by different stages of maturity of the spores, whereby the cingulum did not fully develop until maturity.

Discussion

All individuals belong to a single haplotype on the basis of five plastid markers. This was expected for the gene regions *rbcL* and *rpoC1*, but less so for the usually more variable spacer regions *trnL*-F, *psb*A-trnH and *rps*4-trnAS. No or little variation in markers commonly used for species-level phylogeny of bryophytes has previously been reported, e.g., for species in the moss genera *Bryum* Hedw. (Holyoak & Hedenäs 2006) and *Drepanocladus* (Müll.Hall) G.Roth (Hedenäs 2008). A lack of variation in common phylogenetic markers does not mean that there is only one species. For example,
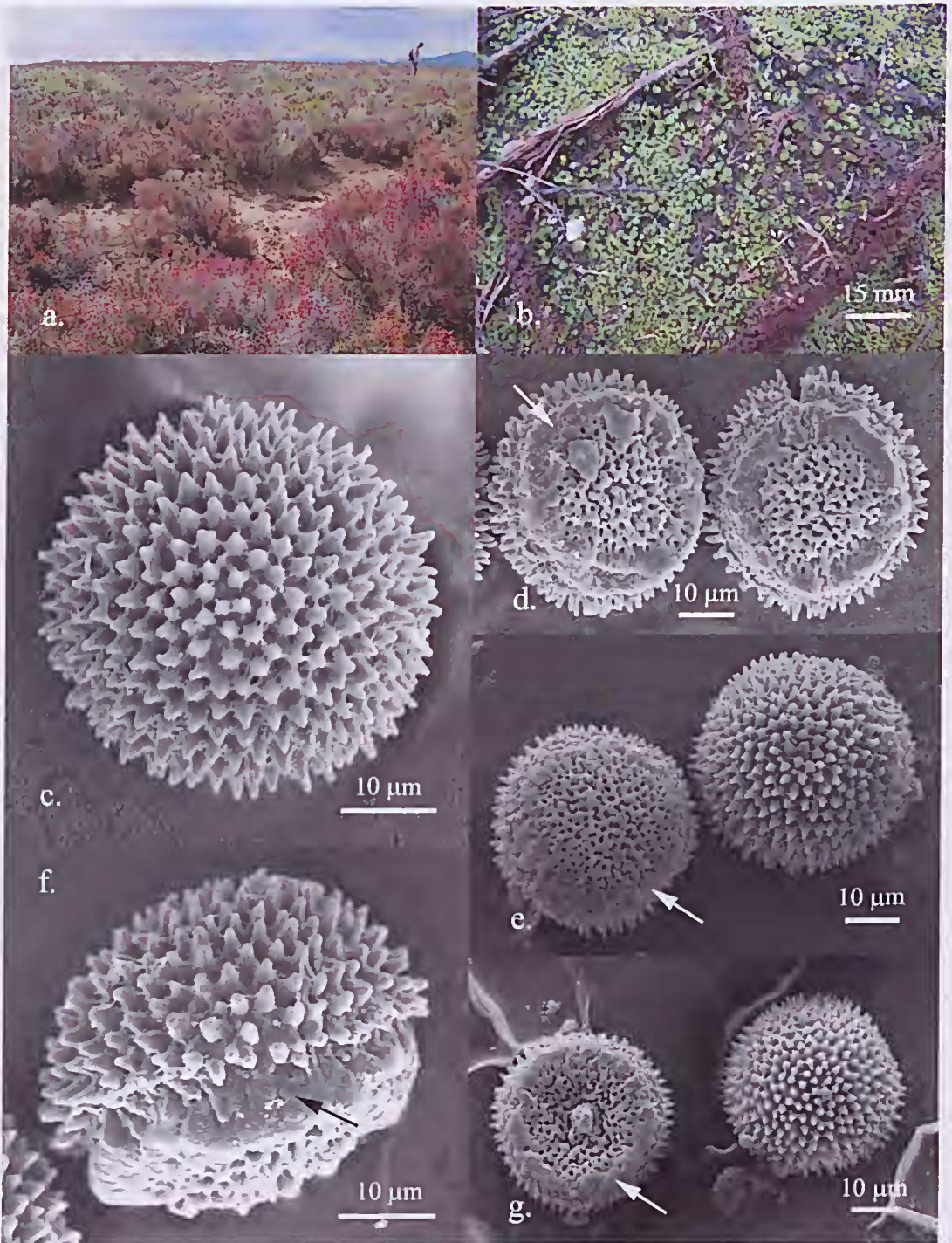
Figure 2. a. Habitat of *Monocarpus sphaerocarpus* at Winninowie CP, South Australia; **b.** plants *in situ* at Port Augusta mud flats, South Australia; **c.** distal view of spore from *Carr s.n.* collections from Victoria; **d.** proximal views of spores from *Carr s.n.* collections from Victoria; **e.** proximal and distal view of spores from Western Australian collections, *Jolley & Milne 171*; **f.** equatorial view of spore showing the cylindrical shape of the proximal cingulum from *Carr s.n.* from Victoria; **g.** proximal and distal view of another two spores from the same capsule as *e.* from Western Australian collections, *Jolley & Milne 171*. All arrows indicate position of cingulum on the proximal face of spore.

in the liverwort *Frullania asagrayana* Mont., although the plastid loci that were sequenced proved invariant, the use of microsatellite markers allowed detection of reproductively isolated groups (Ramaiya et al. 2010).

In contrast, when we looked at within-species sequence variation in a second Sphaerocarpalean genus, *Sphaerocarpos*, only one of the four species sampled had DNA sequences identical for all loci of all accessions (*Sphaerocarpos texanus* Austin from France, Portugal, Turkey and the United Kingdom – no variation for *rbc*L and *rpo*C1, one base change in *psb*A-trnH). Within the three other morphologically defined species we sampled, the sequences from all three loci were variable (*S. stipitatus* Bisch. ex Lindenb. from South Africa and Nepal — one base change in *rbc*L; one base change in *rpo*C1; four base changes in *psb*A-trnH; *S. drewei* Wigglesw. from California, USA — two base changes in *rbc*L; three base changes in *rpo*C1; one base change in *psb*A-trnH; *S. michelii* Bellardi from France, Portugal and the United Kingdom — up to nine base changes in *rbc*L; up to seven base changes in *rpo*C1; up to five base changes in *psb*A-trnH). As well as being phylogenetically related, species in *Sphaerocarpos* share some ecological features with *Monocarpus*, including a tendency to occur in ephemeral habitats.

For *Monocarpus sphaerocarpus*, we consider that the spore differences observed by Forrest et al. (2015) are more than likely due to maturity of spores or natural variation within the species (Figs 2d, e). In combination, both the lack of variation within five plastid loci and the lack of clear spore morphological differences across Australian populations are consistent with the recognition of a single species. The occurrence of a single chloroplast haplotype across Australia is indicative of either the lack of any barrier to gene flow between populations (i.e. long distance dispersal), or recent range size expansion from a small ancestral population. Population-level markers (e.g. microsatellites) would need to be identified to explore the genetic structure among the widely separated Australian populations of *M. sphaerocarpus*.

As currently sampled, this species has a rather disjunct distribution on the Australian continent.

However, it is rarely collected due to its small size and ephemeral nature, and we have limited knowledge of the duration of its colonies and its life history. Of the 37 collections in Australian herbaria, most were collected in late winter to early spring. Only two sites in Victoria, the Raak Plain and Lake Duchembegarra, have been re-visited and the species relocated and recollected after a period of approximately 50 years. One of these sites, the Raak Plain, was re-visited again the following November (2016) with no sign of the plant (Milne, pers. obs.). Therefore, there is evidence that the lifespans of individuals are most likely brief and their occurrence in time and space sporadic, with all collections probably the result of opportunistic encounters. Known populations may not be truly disjunct, but may form part of a continuous distribution range comprising yet-to-be-discovered populations across southern Australia, stretching from western Victoria through South Australia, along southern Western Australia and finishing up along the mid-west coast region of Western Australia near Carnarvon.

Acknowledgements

References

Carr, D.J. (1956). Contributions to Australian Bryology. I. The structure, development, and systematic affinities of

Monocarpus sphaerocarpus gen. et sp. nov. (Marchantiales). *Australian Journal of Botany* **4**, 175–191.

Cox, C.J., Goffinet, B., Newton, A.E, Shaw, A.J. and Hedderson, T.A.J. (2000). Phylogenetic relationships among the diplolepideous-alternate mosses (Bryidae) inferred from nuclear and chloroplast DNA sequences. *The Bryologist* **103**, 224–241.

Fazekas, A.J., Burgess, K.S., Kesanakurti, P.R., Graham, S.W., Newmaster, S.G., Husband, B.C., Percy, D.M., Hajibabaei, M. and Barrett, S.C.H. (2008). Multiple multilocus DNA barcodes from the plastid genome discriminate plant species equally well. *PLoS ONE* **3(7)**, e2802. doi:10.1371/journal.pone.0002802

Forrest, L.L., Long, D.G., Cargill, D.C., Hart, M.L., Milne, J., Schill, D.8., Seppelt, R.D. and Villarreal, J.C. (2015). On *Monocarpus* (Monocarpaceae, Marchantiopsida), an isolated salt-pan complex thalloid liverwort. *Australian Systematic Botany* **28**, 137–144.

Hedenäs, L. (2008). Molecular variation in *Dreponoclodus oduncus* s.l. does not support recognition of more than one species in Europe. *Journal of Bryology* **30**, 108–120.

Holyoak, D.T. and Hedenäs, L. (2006). Morphological, ecological and molecular studies of the intergrading taxa *Bryum neodomense* and *B. pseudotriquetrum* (Bryopsida: Bryaceae). *Journal of Bryology* **28**, 299–311.

Kress, W.J. and Erickson, D.L. (2007). A two-locus global DNA bar-code for land plants: The coding *rbcL* gene complements the non-coding *trnH-psbA* spacer region. *PLoS ONE* **2**, e508. doi:10.1371/journal.pone.0000508

Maddison, W.P. and Maddison, D.R. (2016). Mesquite: a modular system for evolutionary analysis. Version 3.10. <http://mesquiteproject.org>

Nadot, S., Bajon R. and Lejeune, B. (1994). The chloroplast gene *rps4* as a tool for the study of Poaceae phylogeny. *Plant Systematics and Evolution* **191**, 27–38.

Perold, S.M. (1999). Studies in the Sphaerocarpales (Hepaticae) from southern Africa. 1. The genus *Monocorpus* and its only member, *M. sphoerocorpus*. *Botholio* **29**, 225–230.

Ramaiya, M., Johnson, M.G., Shaw, B., Heinrichs, J., Hentschel, J., von Konrat, M., Davison, P.G. and Shaw, A.J. (2010). Morphologically cryptic biological species within the liverwort *Frullonio osogroyona*. *American Journal of Botony* **97(10)**, 1707–1718.

Taberlet, P., Gielly, L., Pautou, G. and Bouvet, J. (1991). Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* **17**, 1105–1109.

Wynns, J.T. and Asmussen-Lange, C.B. (2014). A comparison of 16 DNA regions for use as phylogenetic markers in the pleurocarpous moss genus *Plogiothecium* (Hypnales). *American Journal of Botany* **101(4)**, 652–669.